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Enzymatic pretreatment of steam-exploded birch wood for increased biogas production and lignin degradation

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ABSTRACT

Lignocellulose is readily available biomass for biogas production; however, due to its rigid structure, it requires pretreatment to obtain a maximum energy extraction. In this study, steam explosion (SE) (220 °C and 10 minute retention time) has been employed to increase the biogas production potential from birch wood. Although the biogas production increased by over two times after SE, the SE of birch wood negatively affects the structure of C_5/C_6 sugars and doubled the concentration of non-degradable lignin in all the samples. In this work, SE birch wood has been further pretreated by novel lignin-degrading enzymes cocktail to convert lignin into degradable sugars and increase the biogas production rate. The proposed hybrid pretreatment could increase the biogas production by up to 25% (from 450.5 mL/g VS to 566 mL/g VS), and reduced the lignin concentration by up to 48%.

1. Introduction

Industrial development has made human societies more dependent on energy in a way that the global energy consumption is increasing. The total global energy supply became greater from 420 EJ in 2000 to 598 EJ in 2018 (World Bioenergy Association (WBA), 2020). Apart from global demand, utilisation of the non-renewable energy resulted in catastrophic problems in global warming. The share of renewable energy sources in different end use sectors including electricity, heating and transportation was 17% in 2018 that was 58% more than that in 2000 (World Bioenergy Association (WBA), 2020). To avoid reaching a tipping point in the climate system (i.e., irreversible point in global warming), a fast switch to renewable energy sources is needed. Bioenergy production including anaerobic digestion (AD), can be act as a reliable shifting tool in this process. In 2018, the global biogas production reached 1.39 EJ that is an extreme increase compare to 0.28 EJ in 2000 (IEA, 2020; Ian Tiseo, 2021). It is also predicted that by the end of 2050 the global biogas production potential from lignocellulosic material can reach up to 100 EJ (Haberl et al., 2010).

In 1990s, first-generation feedstocks such as maize, grasses, cereals, beets, potatoes and sunflowers were cultivated in Germany and Austria as substrates for AD due to their high potential for the biogas production (Murphy et al., 2011; Seadi et al., 2013). First-generation feedstock can be considered as a food source making them less attractive source for bioenergy production (Allen et al., 2016). The second-generation feedstocks such as lignocellulosic materials do not compete with food production, are readily available sources, and have a large carbon content. These turn lignocellulosic biomass into a suitable source for biogas production (Allen et al., 2016). Lignocellulose is composed of cellulose, hemicellulose and lignin. The ratio of these three components varies in lignocellulosic materials depending on several factors including the clade which the plant belongs to (i.e., conifers (angiosperms), dicot angiosperms (hardwoods) or monocot angiosperms (grasses), species and age) (Chen, 2015; Kainthola et al., 2019). Cellulose is a glucose homopolymer that has been tightly glued together with hemicellulose (Bai et al., 2019). Hemicelluloses are a group of polysaccharides found in lignocellulosic material. There are both hexosans (predominantly mannose, glucose, and sometimes galactose) and pentosans (predominantly xylose, arabinose and glucuronic acid) which are shielded by lignin (Brunner, 2014). Lignin is an amorphous phenolic polymer with no obvious repeating structure. The skeleton of its monomers is the phenylpropane structure (Xu, 2010). The combination of these materials

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makes the structure of lignocellulose resistant to biodegradation (Hernández-Beltrán et al., 2019).

In order to extract the degradable sugars from lignocellulosic materials, a pretreatment is needed to change its structure (Hashemi et al., 2021). Several pretreatment methods including acid and alkaline pretreatment, torrefaction, cavitation, liquid hot water extraction, ammonia fiber explosion, wet oxidation, enzymatic pretreatment and steam explosion (SE) have been developed (Hashemi et al., 2021; Tursi, 2019). The main purpose of the pretreatment is to: increase the accessible surface area for enzymatic degradation (i.e., Hemicellulose and lignin degradation leads to increased porosity); Lignin degradation can reduce irreversible binding of cellulase to lignin, resulting in enhanced enzymatic activities and cellulose degradation; and, provide a water-soluble substrate (Hosseini Koupaie et al., 2019; Hashemi et al., 2021). SE is a thermal pretreatment method through which the structure of the lignocellulosic materials is disrupted using high temperature and pressure (Lizasoain et al., 2016). High pressure steam penetrates into the pores of lignocellulosic components. Then after a specific residence time, the substrate is exposed to the atmospheric pressure leading to a sudden expansion due to pressure release. This process breaks down the structure of the lignocellulosic materials (Horn et al., 2011b). It has been reported that the higher severity factors (see Section 3.1) turns the lignocellulose to sticky fine particles with less observable fibers in darker colors (Ballesteros et al., 2004; Horn and Eijsink, 2010). It has been previously shown that the SE is an effective pretreatment method that can significantly increase biogas production yield from hardwood (willow, salix and birch wood) (Horn et al., 2011a, 2011b; Vivekanand et al., 2013). Along with physical disruption of the lignocellulosic materials, different types of enzymes are capable of degrading various compositions of the lignocellulosic materials (Mirmohamadsadeghi et al., 2021). Cellulases, endoglucanases, b-Glucosidases can degrade cellulose to glucose (Hashemi et al., 2021). Xylanases are enzymes that provide sugar xylan by degrading hemicellulose and consequently, several hydrolytic enzymes (e.g., endo-1,4-b-xylanase, b-xylosidase, a-arabinofuranosidases and esterases) convert xylan to sugar. Lignin degrading enzymes can reduce lignin to cation radicals (Eibinger et al., 2014). The most effective enzymes for lignin degradation are lignin peroxidase, manganese peroxidase and laccase that can be produced from bacteria and white-rot fungi (Hasunuma et al., 2013; Schroyen et al., 2014). The main application of these enzymes is to degrade lignin and to enhance the activity of the cellulase without affecting the hydrolysis process in anaerobic digestion (Lugani et al., 2020).

Firewood has been the main heating source in European houses for years because forestry biomass including hardwoods is readily available in Northern Europe (Vivekanand et al., 2013). In the last decades, wood consumption as the household heating source in Northern European countries has been reduced (Lillemo and Halvorsen, 2013). New heating technologies, zero-emission building (ZEB) concepts and more efficient isolating material had a major role to play in this sector (Knight and Rosa, 2012; Schueftan and González, 2013). In 2015, 168.6×10^6 m^3 solid wood has been consumed in Europe as the energy source in the household sector. By developing ZEBs solid wood can be considered as the potential source of bioenergy (UNECE, 2019; Glasenapp et al., 2020). The wood-based biomass is considered as a CO_2 neutral source since the CO_2 released by using these sources can be absorbed by growing new plant (Holtsmark, 2012); however, this may not applicable for plants used as source of biomass. This is because of the long rotation time (i.e., the period that the CO_2 released from the biomass consumption will be remained in the atmosphere) needed during the growth of the new plant. In a long rotation time, the CO_2 released from biomass consumption may contribute in global warming (Holtsmark, 2012). In case of using plants with shorter growth period, released CO_2 can be consumed by the growing plant turning these biomasses a CO_2 neutral source. Among different sources, birch wood is an available source with short retention period (15–20 years) and high yield (i.e., 3 tons dry weight per ha per year) making it an attractive candidate for biofuel production (Eriksson and Johansson, 2006).

Even though the SE of the birch wood increases biogas yield through an increase of accessible surface area for enzymatic activities, there is still a large amount of carbon content (i.e., particularly from lignin) in the birch wood is unutilized (Vivekanand et al., 2013). In high temperature SE, the C5 and C6 sugars (e.g., xylose and glucose) that are produced during the hydrolyzation of lignocellulose degrade to 5-hydroxymethylfurfural (5-HMF) and furfural. These elements further polymerize making a Humin-like structure (Aarum et al., 2018). These structures appear as lignin in Klason lignin analysis increasing the amount of the unwanted solid fraction after anaerobic digestion.

A hybrid pretreatment including SE and enzymatic pretreatment may further degrade the lignin and cellulose leading to higher biogas yield and reduce the volume of the remaining biomass after anaerobic digestion. The enzyme can be obtained from industrial biotechnological production methods or can be produced in the biogas production plant with lower costs. Table 1 lists recent studies that have been performed on saccharification of lignocellulosic material by steam explosion, enzymatic pretreatment as well as a hybrid pretreatment; however, little work has been done on developing an effective pretreatment in which a maximum saccharification is obtained while the lignin content is reduced. Therefore, the main focus in this study is to develop a hybrid pretreatment including SE and enzymatic pretreatments to increase the biogas production yield as a result of saccharification and reducing the concentration lignin in the samples.

2. Materials & methods

2.1. Raw material

Birch wood, also known as *Betula pubescens* (harvested in Norway), was used as the raw material. The birch wood was shredded into chips of 15 to 30 mm. The wood chips were kept at room temperature for 12 days to reduce their moisture content. Table 2 shows the physical and chemical characterization of the birch wood. The total solid (TS) or dried material (DM) of the birch wood was 81 ± 2. Volatile solids (VS) and lignin content of the total solid were, 87.6 ± 0.3 and 20 ± 2%, respectively (APHA, 2005).

2.2. Steam explosion pretreatment

The SE pretreatment was conducted at the Norwegian Institute of Bioeconomy Research (NIBIO) in Ås, Norway according to the method described previously (Horn et al., 2011b; Vivekanand et al., 2013). A SE rig with maximum load of 10 kg substrate designed by Cambi AS (Asker, Norway) was employed in this study. Before SE, the vessel was pre-heated for 10 min at 220 °C. For SE, the vessel was filled by 1 kg of birch wood chips. The wood chips were subjected to 18–20 bar pressure at 220 °C with a residence time of 10 min. At the final step, the outlet valve was opened to push treated substrate to atmospheric pressure leading to disruption in the substrate structure. The steam-exploded birch wood (SEBW) was packed in a vacuum bag and stored in −20 degree to avoid any microbial activity. The hard shape of the birch wood chips changed after the steam explosion making it sticky brown dough with small fibers. Table 2 shows the physical and chemical characterization of the SEBW. The TS and VS (dry based) of SEBW were 43.3 ± 0.1 and 86 ± 0.3%, respectively (APHA, 2005).

2.3. Inoculum

The microbial inoculum for the biomethane potential experiments was obtained from the Biokraft biogas plant (Skogn, Norway), from a large-scale continuous mesophilic multifuel anaerobic digester that has been used for protein- and fat-rich substrates (Li et al., 2016). Prior to the biomethane potential test, the inoculum was incubated at 40 °C to reduce endogenous biogas production. The TS and VS of the inoculum

Table 1
Main focus area of recently published research papers and the novelty of the current study.

Substrate	Pretreatment	Biogas yield improvement %	Pretreatment effects on structure	Source
Cellulose	SE + LPMO+H_2O_2	2% reduced	Reduced the biogas production yield because of use of LPMO in AD	(Costa et al., 2019)
Milled spruce	SE + LPMO+H_2O_2	52%	No change in lignin content	(Costa et al., 2019)
Birch wood	SE + LPMO+H_2O_2	60%	No change in lignin content	(Costa et al., 2019)
Corn cob	Endo-1,3(4)-β-glucanase, cellulose, xylanase and feruloyl esterase	14.6%	Reduced the lignin content by 10%	(Pérez-Rodríguez et al., 2016)
Wheat straw	SE + celluclast	57%	94% degradation of the chemical oxygen demand (COD)	(Nkemka and Murto, 2013)
Birch wood	SE+ cellulolytic bacterium	21–44%	Improved hydrolysis step, increased in lignin content	(Mulat et al., 2018)
Birch wood	SE + cellicCTec2	Up to 75%	Increased in lignin content, up to 97% increase in glucose	(Vivekanand et al., 2013)
Mixed hard wood	SE+ cellulase, xylanase,		71% increase in sugar content	(Horn and Eijsink, 2010)
Salix	SE + celluclast	Up to 70%	Increase in lignin content	(Horn et al., 2011a)
Reed biomass	SE	Up to 89%	Increase in lignin content	(Lizasoain et al., 2016)
Birch wood	SE+ delignifying enzyme cocktail	Up to 26%	Reduced the lignin content by up to 48%, reduced the solid mass content by up to 38%, Increase in concentration of degradable carbohydrates	Current study

Table 2
Physical and chemical characterization of the birch wood and steam exploded birch wood.

Sample	Size mm	TS%	VS%[a]	Lignin %[a]	Glucose[b]	Xylan[b]	Arabinan[b]	Galactan[b]	Manan[b]	Cellobiose[b]
Birch wood	15–30	81	87.6	20	2.3	0.024	0	0.01	0.13	0.583
Steam exploded birch wood	<4	43	86	52	13.4	11.6	0.4	0.9	0.05	0

[a] The volatile solid and lignin content presented based on the dry matter content.
[b] Sugar content has been measured based on complete saccharification of 10 g substrate.

was 4.3 and 57%, respectively, with a pH of 7.5.

2.4. Enzymatic pretreatment

Enzymatic pretreatment conducted in triplicate within a 50 mL screw-capped test tube with a working volume of 20 mL. For enzymatic pretreatment of each sample, 1 g of SEBW was mixed with 20 mL of distilled water giving a pH of 3.5 ± 0.1. Prior to the enzymatic pretreatment, the pH of samples was adjusted to 7.5 by adding NaOH (4 mol solution). four different commercial enzymes from MetGen (Kaarina, Finland) were used for enzymatic pretreatment, as shown in Table 3 (Horn and Eijsink, 2010; Müller et al., 2018) Enzyme dosages of 10, 20, 50 and 100 μL/g SEBW were investigated within two different incubation time (i.e., 6 and 24 h). After adding the enzyme, the tube cap was half-opened to ensure sufficient airflow to the test tube. The test tubes were shacked by hand every 1 h for 3 min to provide a homogeneous sample. At the end of the incubation process, the air in the tube was flashed out by nitrogen gas for 40 s and then the samples were stored at −20 °C before further use. The frozen samples were located at the laboratory temperature for 2 h to reach room temperature before anaerobic digestion.

Table 3
List of commercial enzymes used in this study.

Supplier	Enzyme code	Exp. code
METGEN	MetZyme FORCI 017	A
METGEN	MetZyme FORCI 021S	B
METGEN	MetZyme FORCI 018	C
METGEN	MetZyme FORCI 032	D

2.5. Biomethane potential

100 mL gas sealed medical syringes were used in triplicate as the batch reactors, as presented in Fig. 1, to investigate the biomethane potential (BMP) test of the enzymatic pretreated samples. One batch with only inoculum alone was used as a negative control as described by Østgaard et al. (Østgaard et al., 2017). The air was removed from the syringes and an airtight on-off valve was installed to the head of the syringes to remove and analyses of the produced gas. The syringes were filled by 40 mL inoculum and 20 mL proceed sample kept at 40 °C for 43

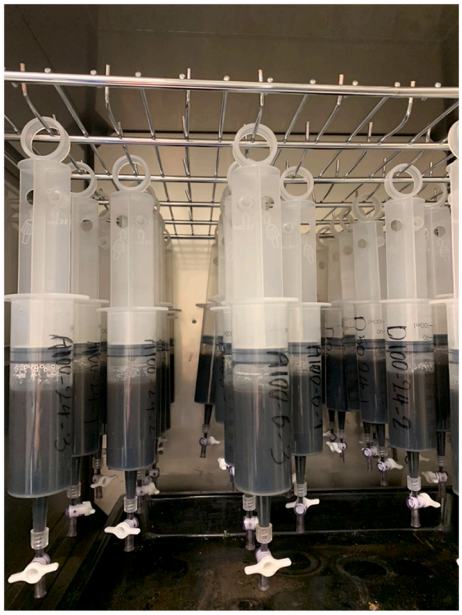

Fig. 1. 100 mL medical syringes have been used as batch anaerobic digester.

days. Content of the syringes were mixed regularly by simply shaking the syringes (Nikbakht Kenarsari et al., 2020).

2.6. Analysis

2.6.1. Composition analysis

Total solids (TS) and volatile solids (VS) were measured using standard protocol ALPHA (American Public Health Association, 2005). For TS, 100 g of wet samples (i.e., triplicated samples) were kept at 105 °C in heating cabinet (Termaks AS, Norway) for 24 h. The dried sample amount was compared with the wet sample and the TS was calculated by Eq. (1) (Sarker, 2020).

$$TS = \frac{\text{dried sample weight} - \text{glass tray weight}}{\text{wet sample weight} - \text{glass tray weight}} \quad (1)$$

Mass of VS determined through the mass difference between TS and ash contents. For this purpose, the dried sample was combusted at 550 °C for 4 h using a muffle furnace (Nabertherm, Germany). The VS was then calculated using Eq. (2) (Sarker, 2020).

$$VS = \frac{\text{combusted sample weight} - \text{glass tray weight}}{\text{dried sample weight} - \text{glass tray weight}} \quad (2)$$

2.6.2. Gas production volume

Gas production volume was achieved by reading the amount of accumulated gas in the syringe through the syringe volumetric (mL) scaling lines at 24 °C (Østgaard et al., 2017; Nikbakht Kenarsari et al., 2020).

2.6.3. Gas composition and calculation

The gas composition analysis was conducted two times for each experiment. 20 mL of gas was collected in a syringe for composition analysis. The gas composition was analyzed by a gas chromatograph (GC) (SRI 8610C, SRI Instruments, USA), equipped with a thermal conductivity detector using nitrogen as the carrier gas. A standard mixture of CO_2, CH_4, H_2 was used as a calibrating gas. All methane production levels are the average of 3 separate experiment after reducing the negative biogas production from inoculum only control experiment.

2.6.4. Volatile fatty acid content

Volatile fatty acids (i.e., VFAs, including acetate, propionate and butyrate) measurements carried out by using a AutoSystem XL (Perkin Elmer) GC machine with autosampler. For VFA measurement, 2 mL of sample was collected from syringe and after centrifuging the sample at 15000 rpm using Microfuge 20 (Beckman Coulter) for 10 min, the sample was filtered with a 0.45 μm filter (514-0071, VWR). The sample was then diluted 4 times with distilled water to be used in GC machine.

2.7. Solid hydrolysation and compositional analyses

Based on the results from biogas production improvement (Section 3.2), the most efficient enzymes were selected. The SEBW was employed to assess the solid solubilisation and lignin degradation during the enzymatic pretreatment. For this purpose, 10 g of the SEBW were mixed with 100 mL of distilled water. Enzyme B and C were employed in four different dosages (i.e., 10, 20, 50 and 100 μL/g SEBW) and incubated for 6 and 24 h as described in Section 2.4.

2.7.1. Solid hydrolysation

Dry matter of the initial sample was weighted. After enzymatic pretreatment, the water was separated by vacuum filters with a 0.45 μm filter paper. After separating effluent liquid, the solid was dried in the room temperature to achieve a moisture content of 3.4 ± 0.7%. The dry matter after tr (Martin-Sampedro et al., 2012; Pérez-Rodríguez et al., 2016):

$$\text{Massloss}\% = 100 \times \left(\frac{\text{Initialdrymatter} - \text{drymatterafterenzymaticpretreatment}}{\text{Initialdrymatter}} \right) \quad (3)$$

2.7.2. Compositional analyses

The lignin and carbohydrate analysis of biomass were carries out using NREL method. For lignin content measurements, the experiments have been run in duplicate. The Klason lignin was calculated by gravimetric method and the acid soluble lignin was qualified by UV/Vis spectrometry at a wavelength of 205 nm (Sluiter et al., 2006, 2008). The enzymatic pretreatment effluent was analyzed by NREL method. The carbohydrates in the effluent were analyzed by HPLC. A Shimadzu Providence HPLC system provided with RI and multichannel UV-VIS detectors was used for analysis with an Agilent Hi-Plex Pb column (300 mm × 7.8 mm) with an inline deashing column (Bio-Rad Micro-Guard) using DI water as mobile phase. The flowrate was 0.6 mL/min and the column temperature was 50 °C. The carbohydrates were detected on the RI detector while the furans were detected by UV-VIS detector.

3. Results and discussion

3.1. Steam-exploded birch

The structure of birch wood chips was disrupted trough SE process at 220 °C for 10 min. The reaction ordinate (R_o) is a single value that combines temperature and residence time through which the severity of the SE process can be calculated as $\log R_o$ (i.e., known as severity factor). This has been shown previously by Vivekanand et al. (2013) that the severity factor of 4.2 to 4.8 can significantly increase the biomethane production rate of birch wood (Vivekanand et al., 2013). Therefor severity factor of 4.5 (i.e., 220 °C for 10 min) has been selected for this study. As a result of SE, the final product had dark brown color and the fibers were converted to smaller particles; however, as shown in Fig. 2 some small fibers were observed. There was a risk of losing some of volatile organics during the pressure release step that could lead to lower biogas production potential of the final samples.

Fig. 2. SEBW. 30 mm birch wood chips were converted to a sticky, low fiber residue of dark brown color.

3.2. Biomethane potential of steam-exploded birch wood

As a baseline for biomethane production potential (BMT), an experiment was undertaken using SEBW as the control. BMT test for the control was conducted in triplicate parallel samples simultaneously with other experiments for 43 days. The result from BMP test of the control is provided in the Table 6. Total biogas production from SEBW reached 450 mL/g VS. Considering the methane content of the biogas obtained from SEBW (57%) the biomethane content (i.e., the methane component of the raw biogas) of the SEBW was 256.8 mL/g VS. Even though this was a significant improvement in biogas production from birch wood chips without any pretreatment (i.e., 176 mL/g VS, the data is not presented here), SEBW in this experiment produced lower biogas compared to similar severity factors that were reported by Vivekanand et al. (2013) (340 ± 20 mL CH_4/g VS) (Vivekanand et al., 2013). This could be due to several factors including the type of inoculum used for the experiment, release of volatile organics during SE, and the type of wood used. The biomethane content of enzymatic pretreated samples could increase up to 345 ± 18 mL CH_4/g VS. Biogas production from the SEBW at 220 °C for 10 min still had higher production rate observed for Salix (240 mL/g VS) pretreated by same facility (Horn et al., 2011a). Since SE increases the accessible surface area for enzymatic pretreatment, the finer the particle, the higher the biogas yield. It was also observed that steam-exploded birch saw dust has higher biogas yield and higher production rate (data are not presented here) rate compare to wood chips. Hence, a potential correlation between the particle size and biogas yield could be derived from these observations (Gallegos et al., 2017; Simangunsong et al., 2020).

3.3. Biomethane potential of enzymatic pretreated substrate

Four different enzymes were employed to conduct enzymatic pretreatment on the SEBW as described before. One gram of the SEBW was mixed with 20 mL of water and the pH then was adjusted to 7.5 prior to adding the enzyme. pH 7.5 has been recommended by supplier as the optimum pH for lignocellulosic based material. The enzymes can actively decompose the lignocellulosic materials in presence of oxygen reagent; therefore, an air flow was supplied to the test tube during the incubation time. Four different dosages were incubated for 6 and 24 h. Enzyme dosage and incubation time of all samples have been shown in the Table 4. The final substrate after the enzymatic pretreatment was a light brown watery mixture with settleable sludge-like solids. After the pretreatment, the air was flashed from the test tubes and the samples were kept at −20. The VFA content observed in these experiments is shown in Table 5.

3.3.1. BMP test of enzymatic pretreated samples

Prior to the BMP test, all frozen samples were in the laboratory at the ambient temperature for 2 h to melt and to reach the room temperature. Table 6 presents the biogas production and the amount of biomethane obtained from these samples during the 43 days. According to the gas composition analysis results that are presented in Table 6 and compared to the experiment with only SEBW, the methane content of all the samples after enzymatic pretreatment increased. The maximum condition reached 61% in the experiments using enzyme B and C. The maximum biogas production using enzyme A was 465.9 ± 26.6 mL/g VS (WA5024) which is 3.33% higher than biogas production from the control. In the optimum condition, maximum biogas production from the experiment using enzyme D increased by 8% (i.e., compared to control) and reached 486.1 ± 21.2 mL/g VS (WD10024).

Enzyme B and C had the best performance in terms of biogas production improvement. The maximum biogas produced from samples pretreated by enzyme B and C were 556.3 ± 3.5 (WB1006) and 566 ± 29 (WC10024), respectively. These are 23.6 and 25.8% higher than biogas

Table 4
Designed parameters for experiment set-up.

Substrate	Name of enzyme	Simple ID	Enzyme dosage (μL/g SEBW)	Incubation time (h)	Inoculum (mL)	Substrate (g)
Inoculum	–	I	–	–	40	0
SEBW	–	W	–	–	40	1.1 ± 0.05
SEBW and enzyme A	FORCI 017	WA106	10	6	40	1.02
		WA1024	10	24	40	1.02 ± 0.02
		WA206	20	6	40	1
		WA2024	20	24	40	1.02
		WA506	50	6	40	1.02
		WA5024	50	24	40	1.05 ± 0.3
		WA1006	100	6	40	1.01
		WA10024	100	24	40	1.06 ± 0.02
SEBW and enzyme B	FORCI 021S	WB106	10	6	40	1
		WB1024	10	24	40	1
		WB206	20	6	40	1.06 ± 0.01
		WB2024	20	24	40	1.04
		WB506	50	6	40	1.02
		WB5024	50	24	40	1.06 ± 0.02
		WB1006	100	6	40	1.02
		WB10024	100	24	40	1.04 ± 0.02
SEBW and enzyme C	FORCI 018	WC106	10	6	40	1.05
		WC1024	10	24	40	1
		WC206	20	6	40	1.02
		WC2024	20	24	40	1.02 ± 0.02
		WC506	50	6	40	1
		WC5024	50	24	40	1
		WC1006	100	6	40	1.02
		WC10024	100	24	40	1
SEBW and enzyme D	FORCI 032	WD106	10	6	40	1
		WD1024	10	24	40	1
		WD206	20	6	40	1
		WD2024	20	24	40	1.05 ± 0.03
		WD506	50	6	40	1.06 ± 0.4
		WD5024	50	24	40	1
		WD1006	100	6	40	1
		WD10024	100	24	40	1

Table 5
Concentration of the volatile fatty acids.

Experiment	Initial pH	VFAs (mg/L)		
		Acetic acid	Propionic acid	Butyric acid
Inoculum	7.5	21.4 ± 1.3	n.d.	n.d.
Control 1	7.48	290 ± 6	39 ± 0.87	n.d.
WA106	7.5	207 ± 10.8	31.7 ± 1.6	n.d.
WA1024	7.5	250 ± 8.7	26.4 ± 1.5	n.d.
WA206	7.51	232 ± 10	23.9 ± 1.5	n.d.
WA2024	7.48	297 ± 7.6	31.7 ± 1.2	n.d.
WA506	7.49	268 ± 8.5	26.9 ± 0.9	n.d.
WA5024	7.51	293 ± 9.1	28 ± 0.8	n.d.
WA1006	7.53	253.1 ± 7.9	13.1 ± 1.7	n.d.
WA10024	7,51	282 ± 1.9	27 ± 0.9	n.d.
WB106	7,49	293 ± 19.1	35.1 ± 1.1	n.d.
WB1024	7.5	284 ± 17.6	23 ± 1.2	n.d.
WB206	7.52	285 ± 8.89	24.4 ± 1.7	n.d.
WB2024	7,49	282 ± 6.5	25 ± 0.2	n.d.
WB506	7.5	332 ± 12.7	23 ± 0.5	0.3
WB5024	7.51	296 ± 11.7	32 ± 0.2	n.d.
WB1006	7.48	363 ± 13.9	30 ± 1.1	n.d.
WB10024	7.6	347 ± 7.5	26.4 ± 1.2	n.d.
WC106	7.5	285 ± 11.3	22.1 ± 0.9	0.2
WC1024	7.51	277 ± 3.9	19.5 ± 0.7	n.d.
WC206	7.49	301 ± 4.9	19.6 ± 0.2	n.d.
WC2024	7.52	305 ± 10.3	21.1 ± 0.3	n.d.
WC506	7.5	320 ± 2.2	25 ± 1.8	n.d.
WC5024	7.5	323 ± 6.1	28 ± 0.5	n.d.
WC1006	7.53	346 ± 6.2	29 ± 0.6	n.d.
WC10024	7.52	385 ± 13.5	32 ± 1.6	n.d.
WD106	7.51	268.7 ± 9	23.3 ± 0.8	n.d.
WD1024	7.48	260 ± 9.5	23 ± 0.9	n.d.
WD206	7.49	289 ± 17.2	18.2 ± 1.3	n.d.
WD2024	7.5	250.4 ± 5,1	21.7 ± 1.4	n.d.
WD506	7.53	305 ± 3	28 ± 0.7	n.d.
WD5024	7.47	300 ± 5.5	22 ± 0.7	n.d.
WD1006	7.5	308 ± 8	25.2 ± 1.5	n.d.
WD10024	7.52	313 ± 2.9	27 ± 0.9	n.d.

n.d. = not detected.

production from the control. The lower dosages of enzymes B and C (i.e., 20 and 50 μL) also had higher biogas production compared to the BMP test with only SEBW.

The maximum methane production from samples that were pretreated by enzyme A and D was 279.5 and 291.6 mL/g VS, respectively, which are 9 and 13.5% higher than methane produced from only SEBW. This increase is mainly due to the increased methane content in all the samples. The maximum methane yield from the samples that were pretreated by enzyme B improved by 32.1% and reached to 339.4 mL/g VS. This value for experiments using enzyme C was 345 mL/g VS. These biomethane production levels are close to those reported previously by Lamb et al. (2019) and Vivekanand et al. (2013) (Vivekanand et al., 2013; Lamb et al., 2019). The biomethane production yield from SEBW combined with an enzymatic pretreatment that have been reported here is in higher range of biomethane production with similar methods for different lignocellulosic materials (Hashemi et al., 2021). This considerable improvement through the proposed approach indicates that the annual biogas production yield, in an industrial anaerobic digester with birch wood as the main substrate, could significantly increase with a similar reactor size (Frigon and Guiot, 2010).

3.3.2. *Enzyme dosage and incubation time*

Enzymes A and D had some positive effects on biogas production, but these enzymes, in general, did not contribute to further biogas extraction compared to biogas production from control. The most promising results observed from experiments involving enzyme B and C, therefore, the rate of these experiments have been investigated in detail. The biogas production rate is an important decision-making factor in an industrial-scale biogas production plant. A higher biogas production rate means the overall capacity of the plant increases. From Figs. 3 and 4, the maximum production rate can be calculated by determining the slop of the accumulative biogas production curve vs time. It can be clearly seen from Figs. 3 and 4 that the biogas yield has been increased by increasing the dosage of enzyme B and C, independent from the incubation time. For enzyme A and D, such a visible trend among different dosages was not observed. These results showed the importance of the correct selection of enzyme for the selected biomass. Not all enzymes can show

Table 6
Accumulative biogas and methane of all samples after 43 days.

Experiment	Biogas after 43 days (mL/g VS)	Methane after 43 days (mL/g VS)	Maximum rate (mL/g VS/day)	CH_4 content (%)	CO_2 content (%)
Inoculum					
I	15.8	9.71	0.7	60	40
Control					
W	451 ± 25.9	257 ± 14.7	59.4	57	43
Enzyme A					
WA106	253 ± 12.4	147 ± 7	26.9	58	42
WA1024	355 ± 36.6	206 ± 21	26.3	58	42
WA206	395 ± 18.9	229 ± 11	31.8	58	42
WA2024	463 ± 42	267 ± 24.3	30.3	58	42
WA506	437 ± 30.6	262 ± 18	39.5	60	40
WA5024	466 ± 26.6	280 ± 16	34.7	60	40
WA1006	421 ± 5.4	248 ± 3	33.7	59	41
WA10024	422 ± 11.43	249 ± 6.7	37.1	59	41
Enzyme B					
WB106	454 ± 10.4	268 ± 6	30.3	59	41
WB1024	391 ± 20.6	231 ± 12	25.5	59	41
WB206	433 ± 4.4	260 ± 2	29,8	60	40
WB2024	414 ± 23.7	244 ± 14	25.5	59	41
WB506	516 ± 2.1	310 ± 1	41.4	60	40
WB5024	450 ± 9.1	270 ± 5.4	30.3	60	40
WB1006	556 ± 3.5	339 ± 2	44.2	61	39
WB10024	537 ± 32.1	327 ± 20	41.3	60	40
Enzyme C					
WC106	443 ± 7.5	261 ± 4.2	32.7	59	41
WC1024	435 ± 14.2	257 ± 8	43.8	59	41
WC206	465 ± 4.6	279 ± 3	33.7	60	40
WC2024	467 ± 26.7	280 ± 16	34.2	60	40
WC506	480 ± 19.3	293 ± 10	33.7	61	39
WC5024	478 ± 13.3	287 ± 9	36.6	60	40
WC1006	550 ± 23.2	335 ± 14	40.4	61	39
WC10024	566 ± 29	345 ± 18	44.3	61	40
Enzyme D					
WD106	390 ± 6.9	226 ± 4	32.7	58	42
WD1024	390 ± 5.9	226 ± 3.7	34.7	58	42
WD206	410 ± 6.5	238 ± 4	35.6	58	42
WD2024	377 ± 14	215 ± 8	25.0	58	42
WD506	436 ± 19	253 ± 12	33.7	59	41
WD5024	418 ± 40.5	251 ± 24	30.0	60	40
WD1006	465 ± 27	274 ± 16	31.8	59	41
WD10024	486 ± 21.2	292 ± 12	35.6	60	40

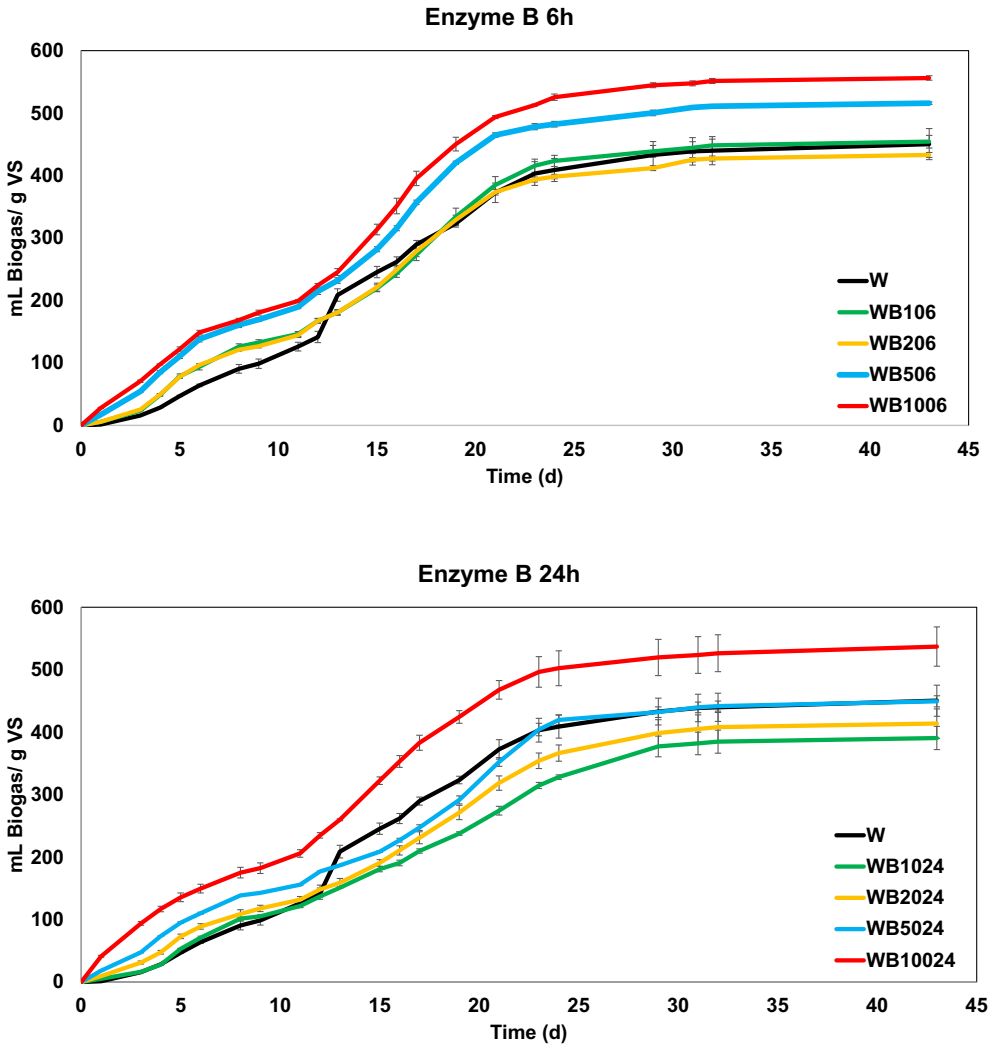

Fig. 3. Biogas production of experiments using different dosages of enzyme B over 43 days. The biogas production of experiments including the control (W) and enzymatic pretreated samples (WB) with 6 h and 24 h incubation time. The error bars are indicative of the extreme spread (i.e., (max-min)/2) of three independent triplicates.

beneficial impact on a process and a careful selection of the activities needs to be done.

As shown in Figs. 3 and 4, in the first week, none of the experiments had a sharp increase in biogas production; however, pretreated samples by enzymes B and C have consistently shown a considerable increase compared to control in the first and third week. Higher biogas yield in the first week of the enzymatic pretreated samples can be due to the degradation of cellulose and maybe lignin components to easily degradable elements (e.g., glucose) as a result of more accessible surface area for effective enzymes. The maximum daily biogas production rate belongs to SEBW which experienced a sudden jump on day 12 (59.4 mL/g VS/day). This is lower than that reported by Vivekanand et al. (2013) in the first week of the experiment. This can be due to the microbial culture used. The inoculum used for this experiment has been collected from a digester adapted for anaerobic digestion of protein-rich substrate leading to a delay in biogas rate at the begging of the experiment. Yet, the diverse culture of the original reactor may ensure faster degradation of the lignocellulosic materials after a specific adaptation period (Poszytek et al., 2017; Ahmed et al., 2019).

In the maximum biogas production condition using enzyme B and C (i.e., 100 μL/g SEBW), the incubation time did not significantly affect the biogas production as indicated in Figs. 3 and 4. For lower dosages a considerable difference can be seen. For enzyme B, an increase of the incubation time from 6 h to 24 h for the samples with 10, 20 and 50 μL/g SEBW resulted in a biomass yield decrease by 12.5 ± 1% while this value for the experiment with 100 μL/g SEBW was only 3.4%. In contrast, the samples that were pretreated by the enzyme C for 24 h had 2.7% higher biogas yield compared to those with 6 h incubation time.

3.4. Lignin and carbohydrate analyses

3.4.1. Mass loss and lignin concentration

To evaluate the mass loss as a result of non-enzymatic activities in different incubation times, two separate samples have been made for SEBW without adding enzyme. The samples were mixed with water and the pH of samples was adjusted to 7.5 by adding NaOH (4 mol solution). These samples were kept at the ambient temperature along with other samples (i.e., samples with enzyme) to investigate the mass lost during the incubation time. After incubation with enzymes B and C, the final substrate was a light brown watery mixture with insoluble sludge-like solids. The watery samples had no visible fibers. After removing the water from the samples and drying the samples at ambient temperatures to reach a moisture content of less than 3%, two random samples were taken from the bulk density (i.e., remaining solid after the pretreatment) for analysis. The samples were taken on the same day to avoid large variation. After Klason lignin, acid-soluble lignin, and sugar analysis, the

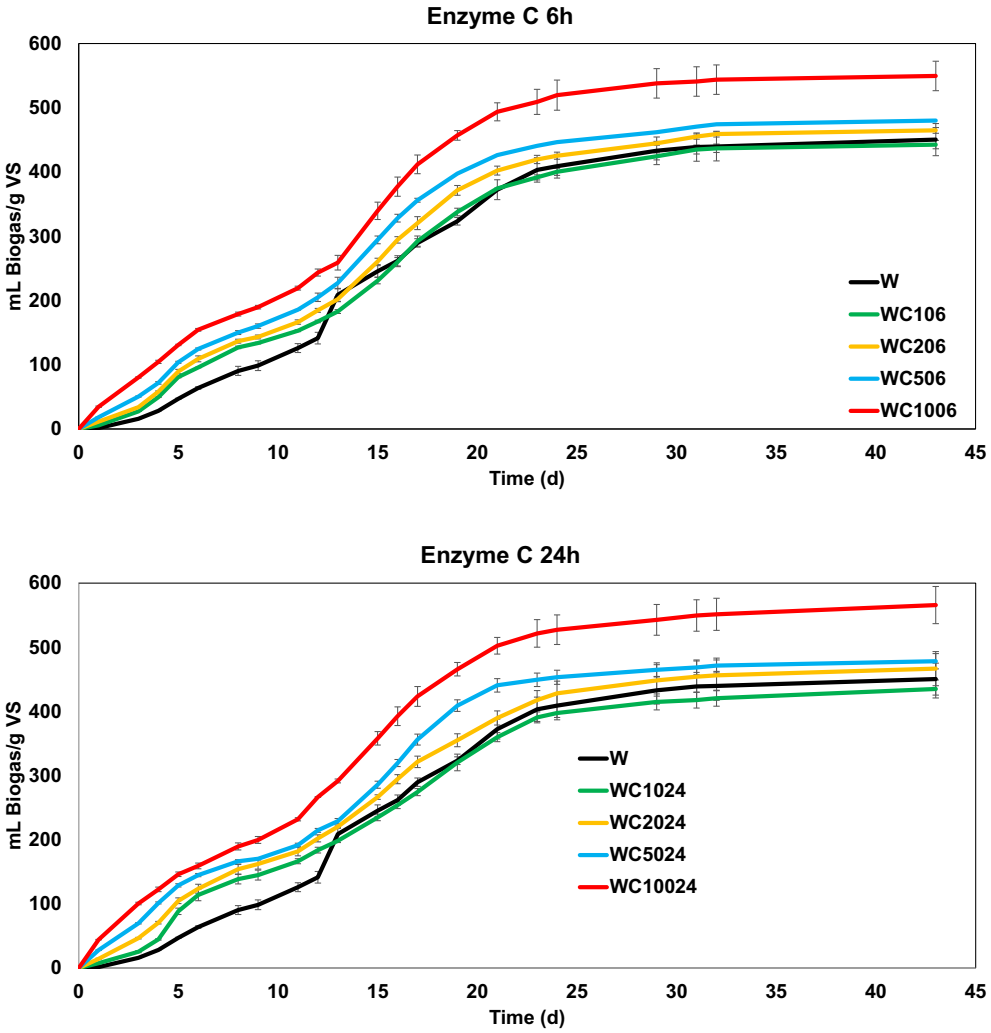

Fig. 4. Biogas production of experiments using different dosages of enzyme B over 43 days. The biogas production of experiments including the control (W) and enzymatic pretreated samples (WC) with 6 h and 24 h incubation time. The error bars are indicative of the extreme spread (i.e., (max-min)/2) of three independent triplicates.

results have been provided in Fig. 5. The average lignin is in fact the average measured total lignin in each sample.

According to the results that presented in Fig. 5, during 6 and 24 h of incubation time, 0.5 and 3% of SEBW mass were lost, respectively. This may cause by the evaporation of volatile solids. These values have been deducted from the mass loss in the other samples. Both enzymes B and C effectively hydrolysed the solids meaning that more soluble organics became available for the microorganisms in AD. It is visible in Fig. 5 that the longer incubation time also results in more hydrolysation of the solids. In 6 h incubation, the maximum mass loss is 19% (B1006) while this value for enzyme C is over 25% (C1006). The highest hydrolysis belongs to B10024 and C 10024 with 31 and 38% mass loss, respectively, after 24 h incubation.

The lignin concentration has been reduced in all the samples compared to the only SEBW (1.8 g/10 g SEBW). For enzyme B and C with 24 h incubation time, the lignin concentration faced with more reduction compared with 6 h incubation time. The maximum lignin degradation with enzyme B was 33% (B10024, 1.19 g/10 g SEBW) while this value for enzyme C was 48% (C10024, 0.93 g/10 g SEBW). It should be noted that lignin degradation doesn't mean an absolute increase in biofuel production. In addition to the generation of the degradable sugars that increase the biogas production yield, lignin degradation may be involved in the generation of phenolic compounds. These products not only are difficult to be degraded in AD but also may contribute to the inhibition of microbial activities (Surendran et al., 2018).

3.4.2. *Sugar content and concentration of inhibitors*

The concentration of the different carbohydrates including glucose, xylose, arabinose, galactose, cellobiose, furfural, and Hydroxymethylfurfural (HMF) is provided in Fig. 6. Glucose and xylose are the main substrate in AD of lignocellulosic biomass that can be converted to methane (Batstone et al., 2002; Ge et al., 2015). The concentration of glucose in lower dosages (10 and 20 μL/g SEBW) of either enzyme is not significantly different from the untreated sample. In contrast, the concentration of glucose and xylose has been significantly increased in higher dosages of the enzyme (50 and 100 μL/g SEBW). This increase is even more visible in longer incubation time (24 h) where the concentration of glucose and xylose has reached 59 and 18 mg/L for C10024 along with 49.5 and 16 mg/L for B10024, respectively. As shown in Fig. 6, these values for only SEBW are 13.3 and 11.6 mg/L, respectively. A high concentration of glucose in the separated liquid may also indicate the capability of the enzymes to reduce the crystallinity of the cellulose and solubilising it to its building element (glucose). A diverse microbial community with an active sugar degrading pathway may lead to higher biogas yield from pretreated samples with higher dosages compared with untreated SEBW (Cirne et al., 2007; Ziemiński et al., 2012; Pérez-Rodríguez et al., 2017). The results of sugar analysis are consistent with the amount of the biogas produced from enzymatic pretreated samples

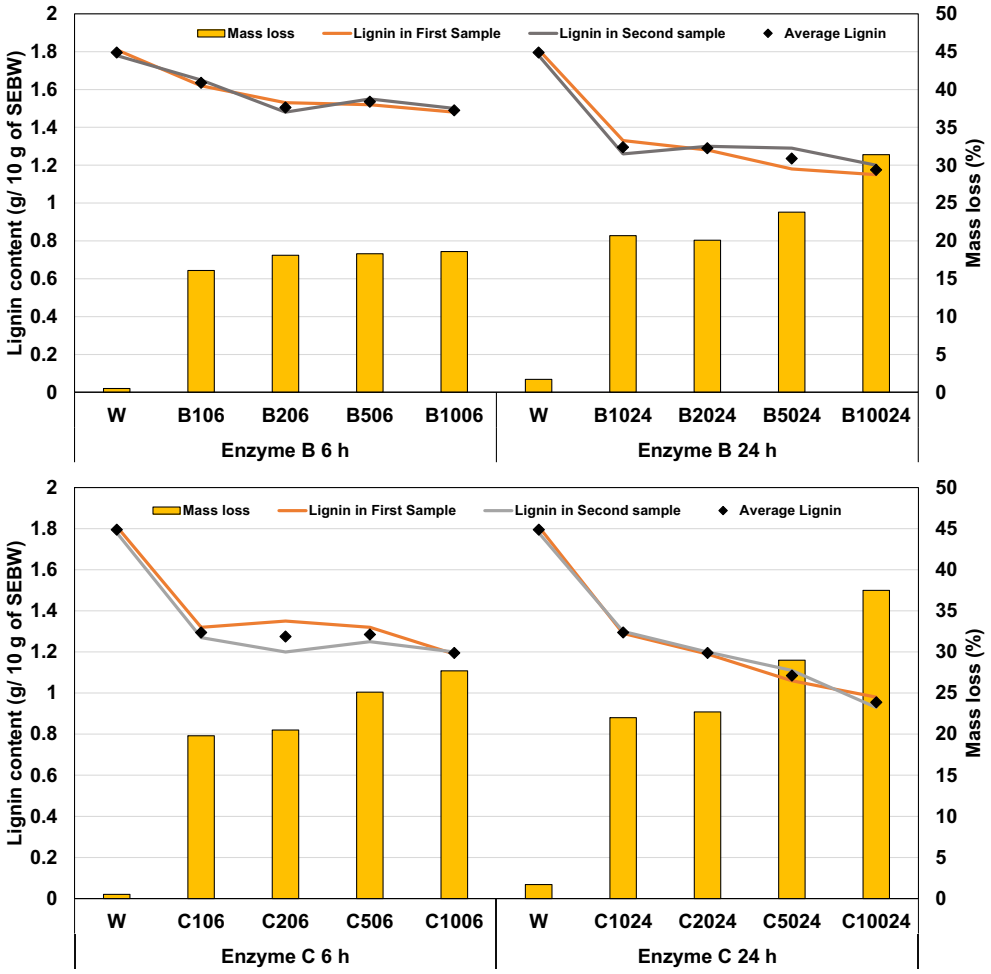

Fig. 5. Lignin concentration after enzymatic pretreatment and the percentage of the mass loss for enzyme B (up) and enzyme C (down). Lignin concentration of each parallel (lines), the average lignin concentration of each sample (black diamonds) and the percentage of hydrolysed solid mass during the enzymatic pretreatment (bars).

as well as the results of the mass hydrolysation and lignin degradation that is presented in this study.

The concentration of the furfurals and HMF has been presented in Fig. 6. The concentration of these elements is not varying notably as a result of enzymatic pretreatment; however, an upward trend appeared by increasing the enzyme dosage. The concentration of total inhibitors in all the samples was 0.42 ± 0.027 mg/L. The HMF is not volatile (Brethauer et al., 2020) and follows the liquid phase and accumulated in the wet sample during steam explosion. After enzymatic pretreatment of the samples, the concentration of furfural and HMF was almost constant around 0.21 ± 0.014 mg/L and 0.2 ± 0.018 mg/L, respectively.

Pure furfural has a boiling point of 161.7 °C (Marcotullio, 2011). According to the furfural/water phase diagram presented by Marcotullio (2011) (i.e., for the pressure range of 1–10 atm) (Marcotullio, 2011), in the low concentrations of furfural in water (<5% wt/wt), the evaporation temperature of the mixture is dominated by the water boiling point (Zeitsch, 2000). The operating pressure for the steam explosion was 19–20 bar; therefore, it can be assumed that the evaporation temperature for the low concentration furfural/water mixture is close to boiling point of pure water at 20 bar (212.3 °C) (National Institute of Standards and Technology (NIST), 2018). This means that in the operating temperature (220 °C) of steam explosion, most of the furfural is discharged to the atmosphere together with the steam. The provided furfural concentration measurement in this study might be insufficient to draw a clear conclusion regarding its inhibitory effects on AD.

4. Future analysis and works

SE can result in substantial variations in composition of the raw materials for example formation of the pseudo-lignin during SE has been observed (Nuopponen et al., 2004). It has been shown that some inhibitor compound including furfural is released during SE of the lignocellulosic material. This inhibitory effect leads to a slower biogas production rate at the beginning or lower biogas yield in general with in the batch tests and in higher concentrations (Hashemi et al., 2021). The culture adaptation has previously used for providing a suitable microbial consortium for a specific feed such as ammonia-rich substrates (Nordgård et al., 2018). Accordingly, an adapted culture for lignocellulosic materials with an active sugar degrading pathway, capable of tolerating high concentration of inhibitors and also high VFA accumulation in the system, may increase the biogas production rate resulting in higher overall capacity of a full-scale plant (Moreno et al., 2016; Nordgård et al., 2017).

It should be noted that SE requires high quality thermal energy. This can be obtained either from the external heat sources (e.g., from the local industrial waste heat) or can be produced internally by burning biogas. On the other hand, the enzyme is an expensive part of the pretreatment that may affect the financial viability of the pretreatment process. Producing enzyme through growing fungi on fresh biomass in the biogas plant could a cheaper solution. Along with the economic study, an advanced sustainability and life cycle assessment is needed to improve the energy conversion system from the viewpoints of

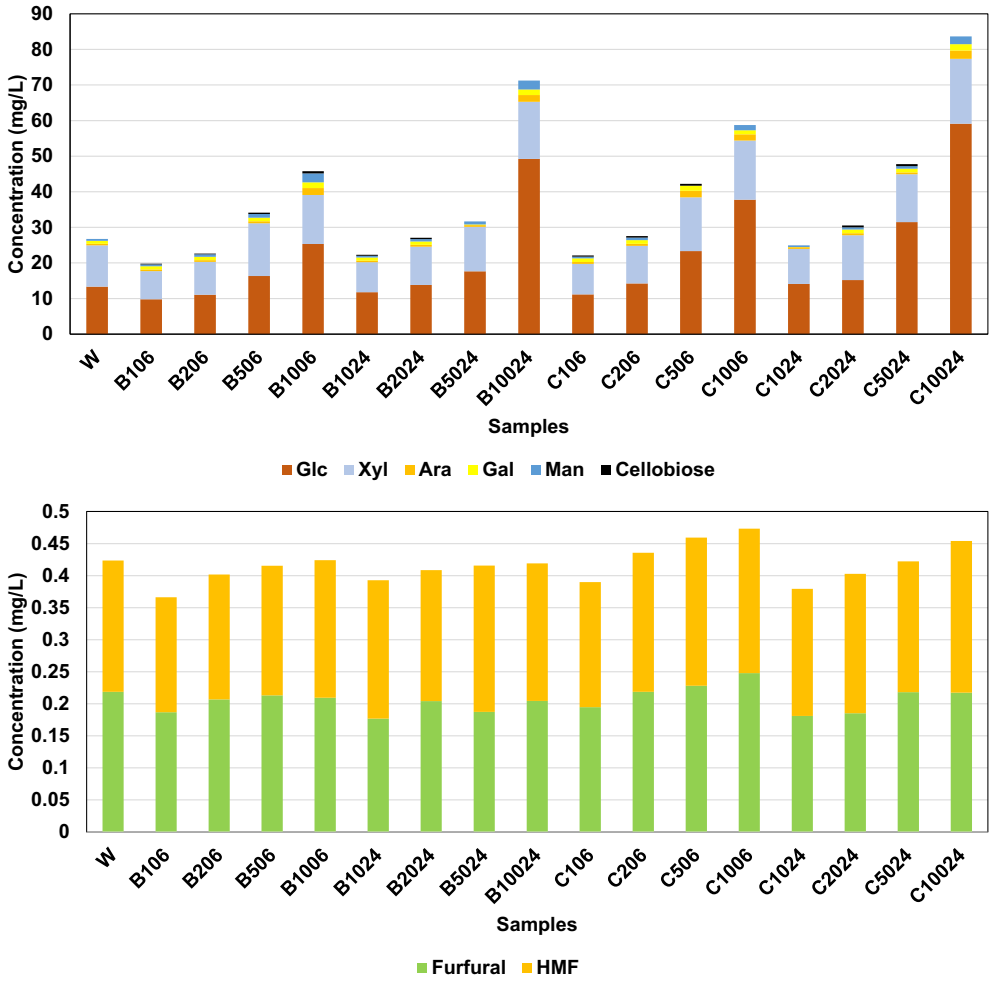

Fig. 6. Concentration of sugars (up) and inhibitors (down) in the separated liquid right after enzymatic pretreatment.

thermodynamics, energy recovery and environmental impacts (Rosen, 2018).

5. Conclusions

According to the results, proposed approach increases biogas yield compared to the unpretreated sample as well as the SEBW sample; however, inefficient enzymatic pretreatment can negatively affect the biogas yield. It was observed that ineffectual enzyme dosage can reduce the biogas production yield in some cases. This indicates the importance of the selection and correct design of enzyme mixtures for specific application. It is shown that efficient use of enzymes increases biogas yield by up to 25.5%. It can also degrade lignin by up to 48% more than SE, and reduces the solid mass by over 30%.

CRediT authorship contribution statement

Seyedbehnam Hashemi: Investigation, Methodology, Formal analysis, Conceptualization, Writing – original draft. **Prajin Joseph:** Formal analysis, Writing – review & editing. **Antoine Mialon:** Writing – review & editing. **Størker Moe:** Validation, Writing – review & editing. **Jacob J. Lamb:** Supervision, Writing – review & editing. **Kristian M. Lien:** Supervision, Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors would like to submit the manuscript with the title of "Enzymatic pre-treatment of steam-exploded birch wood for increased biogas production and lignin degradation" by Seyedbehnam Hashemi, Prajin Joseph, Antoine Mialon, Størker Moe, Jacob J. Lamb & Kristian M. Lien as the authors to be considered for publication as a research paper in the Journal of Bioresource Technology Report.

The scope of this work is to evaluate the effects of a hybrid thermal/biological pretreatment on biogas production yield of birch wood, a readily available lignocellulosic material in Northern Europe. The scope of the paper is well fit with scopes of the Journal of Bioresource Technology Report and the content meets the subject classification (i.e., numbers 20.030, 30.040, 40.010 and 70.090). They declare that this original manuscript has not been published before and is only submitted for the Journal of Bioresource Technology. All financial supports for this project have been supplied by the Norwegian University of Science and Technology through ENERSENSE strategic research program initiative. They confirm that the submission of this manuscript has been approved by all the above mentioned authors.

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